STIC-ILL

QP 251. M554

From: Schnizer, Richard
Sent: Monday, April 14, 2003 8:54 AM
To: STIC-ILL
Subject: 09/873,546

Please send me a copy of:

TI Expression patterns of novel genes during mouse preimplantation embryogenesis.
AU Temeles G L; Ram P T; Rothstein J L; Schultz R M
SO MOLECULAR REPRODUCTION AND DEVELOPMENT, (1994 Feb) 37 (2) 121-9.

Thank you-

Richard Schnizer, Ph.D.
Patent Examiner
Art Unit 1635
CM1 12E17
703-306-5441
Mail Box CM1 11E12

MOLECULAR REPRODUCTION AND DEVELOPMENT 37:121–129 (1994)

Expression Patterns of Novel Genes During Mouse Preimplantation Embryogenesis

GRETCHEN L. TEMELES,[1] PRAHLAD T. RAM,[1] JAY L. ROTHSTEIN,[2] AND RICHARD M. SCHULTZ[1]
[1]*Department of Biology, University of Pennsylvania, and* [2]*Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, Pennsylvania*

ABSTRACT Little is known about the repertoire of genes expressed following zygotic gene activation, which occurs during the two-cell stage in the mouse. As an initial attempt to isolate novel genes, we used previously prepared two-cell and two-cell subtraction cDNA libraries (Rothstein et al., Genes Dev 6:1190–1201, 1992) to isolate a panel of seven cDNA clones. Three cDNAs had no match in the current DNA sequence data banks and three others revealed sequence homology to portions of sequences in the data banks. One cDNA was 90% homologous to the *ras*-related gene *Krev/rap* 1A. The temporal patterns of expression of these genes during oocyte maturation and preimplantation development were analyzed by a reverse transcription–polymerase chain reaction (RT-PCR) assay developed to measure relative levels of mRNAs. Three distinct temporal patterns of expression, designated Classes 1–3, were found. The two Class 1 genes displayed an actin-like pattern, with a gradual decline in expression during oocyte maturation and through the two-cell stage, followed by increases at the eight-cell and/or blastocyst stages. The four genes in Class 2 were expressed at relatively high levels during oocyte maturation and through the one-cell stage and then declined abruptly between the one- and two-cell stages; an increase then occurred at the eight-cell and/or blastocyst stages. The expression of the gene in Class 3 declined during oocyte maturation, but then showed a transient increase at the one-cell stage, with only a very slight increase in synthesis at either the eight-cell or blastocyst stage.

Key Words: Mouse preimplantation embryo, Gene expression, RT-PCR, cDNA

INTRODUCTION

The degradation of maternal mRNA begins during oocyte maturation and is essentially completed by the late two-cell stage. This maternal contribution sustains development of the one-cell embryo to the two-cell stage, at which time the zygotic genome is activated (Bachvarova and DeLeon, 1980; Clegg and Pikó, 1983a,b); α-amanitin does not inhibit the cleavage of one-cell embryos to the two-cell stage, but it does prevent further development of the two-cell embryo. A consequence of zygotic gene activation (ZGA) is the re-expression of transcripts that are common to both the oocyte and embryo, as well as the expression of transcripts that are unique to the embryo. Although the pattern of expression of several "housekeeping" genes is known, for example, actin and tubulin (Paynton et al., 1988), results of experiments examining gene expression by high-resolution two-dimensional gel electrophoresis indicate that ZGA entails a very complex and extensive change in the pattern of gene expression (Latham et al., 1991).

The paucity of biological material has hindered identification and analysis of zygotically activated genes and essentially restricted this analysis to "housekeeping" genes, that is, mRNAs that are usually abundant. Although the advent of reverse transcription–polymerase chain reaction (RT-PCR) has overcome the problem of limiting amounts of embryo RNA available for analysis (e.g., Rappolee et al., 1988), this method is restricted to the analysis of genes for which sequence information is available. Thus, the identification of novel genes and analysis of their function during preimplantation embryogenesis are compromised.

The recent production of high-quality and representative cDNA libraries from mouse preimplantation embryos at different stages of development, as well as the generation of a subtracted two-cell cDNA library, have overcome these problems (Rothstein et al., 1992). Analysis of these cDNA libraries provides a rich opportunity to study differential gene expression in the early mouse preimplantation embryo. Further understanding of the regulation of ZGA would be greatly facilitated by the molecular cloning of genes known to be specifically expressed during this period of development. An example of such stage-specific gene expression is that of the transcription-requiring complex (TRC). The TRC is a family of structurally related proteins of Mr = 70,000 that are associated with the nucleus and whose synthesis, which can constitute up to 42% of total protein synthesis (Latham et al., 1991), is inhibited by α-amanitin and is restricted to the two-cell stage (Conover et al., 1991).

In an effort originally intended to isolate cDNAs encoding the TRC, we developed a screening method that identified clones from the cDNA libraries that con-

Received May 20, 1993; accepted July 6, 1993.
Address reprint requests to Richard Schultz, Department of Biology, University of Pennsylvania, Philadelphia, PA 19104-6018.

tained long open reading frames; such clones would be more likely to encode functional cDNAs than those obtained by random selection. In addition, it was likely that these clones would represent novel genes, since a recent study indicates that about one-third of the cDNAs isolated simply by random picking from a fetal brain cDNA library represent novel sequences (Adams et al., 1991).

We report here the temporal pattern of expression of seven of these cDNAs during oocyte maturation and preimplantation development using RT-PCR. We observe three distinct patterns of expression. The first expression pattern resembles that of previously reported specific mRNAs such as actin, and shows a gradual decrease during oocyte maturation to the two-cell stage, followed by an increase by the eight-cell and blastocyst stages. The second pattern evinced relatively high levels of expression through the one-cell stage followed by a sharp decrease at the two-cell stage and subsequent rise at the eight-cell and/or blastocyst stages. The third expression pattern displays a transient increase in expression that is confined to the one-cell stage.

MATERIALS AND METHODS

cDNA Libraries

The cDNA libraries prepared were as previously described (Rothstein et al., 1992). The two-cell subtraction library was generated by subtraction of the two-cell library against anti-sense transcripts from an egg and eight-cell cDNA library. The cDNA clones were contained in a modified Bluescript (Stratagene) vector (Rothstein et al., 1992).

DNA Sequencing

Double-stranded DNA was sequenced using an ABI Taq Dyedeoxy Terminator Cycle Sequencing kit. Plasmid DNA was prepared for sequencing from cultures that had been grown to saturation overnight on Terrific Broth according to the manufacturer's instructions. Initial DNA purification was performed with a "Magic Miniprep" kit (Promega) according to the manufacturer's instructions and the DNA was precipitated with 13% polyethylene glycol as recommended in the ABI sequencing kit manual. DNA sequencing reactions were prepared exactly as specified by the manufacturer's instructions and cycled on a Perkin Elmer Cetus 9600 thermocycler using thin-walled "Gene-Amp" tubes without mineral oil. The following program was used: A 96°C soak for 10 sec, followed by a cycle program of 25 cycles of 95°C for 10 sec, 50°C for 1 sec, and 60°C for 4 min. The reaction products were purified on G-50 "Select D" spin columns (5 Prime-3 Prime, Inc.) and then the reactions were processed on the Model 373A DNA Sequencing System (ABI).

The 3′ primer and 5′ primer for the initial sequencing were pB-FOUT [5′-GGAAACAGCTATGACCAT-3′] and pB-ROUT [5′-ACGACGTTGTAAAACGAC-3′], respectively; the primers were the generous gift of Holman Massey. The primer pB-FOUT begins three nucleotides 5′ of the M13 reverse primer site on the pBluescript II KS+ vector (Stratagene) and pB-ROUT begins six nucleotides 3′ of the M13(−20) primer on the same vector. Each plasmid was sequenced twice and the sequences were analyzed using the BLAST program (Altschul et al., 1990).

Oocyte, Egg, and Embryo Collection and Culture

Fully grown mouse oocytes freed of attached cumulus cells were obtained from PMSG primed CF-1 mice (Harlan) as previously described (Schultz et al., 1983); 0.2 mM 3-isobutyl-1-methyl xanthine was present in the collection medium to inhibit resumption of meiosis. Ovulated eggs were collected from superovulated mice 19–20 h following hCG administration as previously described (Endo et al., 1987). One-cell, eight-cell, morula, and blastocyst stage embryos were collected from superovulated females that were mated to B6D2F_1/J males (Jackson Laboratory) by flushing either the oviduct or uterus as previously described (Manejwala et al., 1989). Two-cell embryos were obtained by culturing one-cell embryos overnight in CZB medium (Chatot et al., 1989) containing 1 mM glutamine in an atmosphere of 5% CO_2 at 37°C.

The collection medium was bicarbonate-free minimal essential medium (Earle's salts) containing pyruvate (100 μg/ml), gentamicin (10 μg/ml), polyvinylpyrrolidone (PVP) (3 mg/ml), and 25 mM HEPES, pH 7.2 (MEM/PVP).

RNA Preparation

Unless otherwise stated, all solutions were prepared with water that had been treated 0.1% diethylpyrocarbonate.

After collection, oocytes, eggs, or embryos were washed through three drops of MEM/PVP without bicarbonate (Conover et al., 1991), counted, and transferred in a minimal volume to a chilled 0.6 ml microfuge tube on ice. Lysis buffer (100 μl of 4 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 7.4, 1 M 2-mercaptoethanol) was immediately added and the sample vortexed vigorously. *E. coli* rRNA (20 μg) (Boehringer Mannheim) was then added as carrier, the samples vortexed again, and stored at −70°C. (It was possible to thaw and refreeze the mixture once or twice, but the percentage of carrier recovered was significantly reduced upon more repeated freezing and thawing. When multiple collections of embryos were necessary, the samples were frozen in smaller aliquots and pooled for processing.)

Prior to isolation of the RNA, the tubes were thawed on ice and 0.125 pg of rabbit globin mRNA (BRL) was added per embryo; this mRNA served as an internal control for RNA recovery and efficiency of the reverse transcription–PCR reactions (see below). The RNA was precipitated by the addition of 8 μl of 1 M acetic acid, 5 μl of 2 M potassium acetate, and 60 μl of 100% ethanol. The samples were precipitated overnight at −20°C.

The nucleic acid was collected by centrifugation at 10,000 *g* for 15 min at 4°C. The large off-white pellets were then washed once at 4°C with cold 75% ethanol. The final wash was carefully removed with a pipette and the pellets were air dried just to the point of dryness; overdrying was avoided since the resulting pellets were virtually impossible to resuspend. The pellets were resuspended in 20 μl of Resuspension Solution (RS; 40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$). DNA was degraded by incubating the sample with 1 unit of RQ1 DNase (Promega) for 30 min at 37°C. Following the addition of a second volume of RS, the samples were extracted with 40 μl of RS saturated phenol (Amresco). The samples were vortexed, centrifuged for 3 min at 10,000 *g*, and the aqueous phase was transferred to a 0.5 ml microfuge tube. Five microliters of 3 M potassium acetate, pH 5.2, and three volumes of 100% ethanol were added and the RNA precipitated overnight at −20°C.

The RNA was recovered by centrifugation as described above. The supernatant was removed, and the pellet incubated for 10 min in 75% ethanol at room temperature. The samples were centrifuged for 15 min and the very small, glassy pellets were air dried just to dryness. The pellets were resuspended in water containing two units of RNasin/μl at 25 embryo equivalents/μl. The amount of RNA recovered was determined by measuring the absorbance of an aliquot at 260 nm. Recoveries usually ranged from 85–100%, and any samples that fell below this range were not used for RT-PCR.

Reverse Transcription

Reverse transcription was conducted on 200 oocyte/egg/embryo equivalents. The reactions were carried out in 40 μl of 25 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$ containing 1 mM each of dATP, dGTP, dCTP, dTTP, 10 mM dithiothreitol, 1 unit/μl of RNasin, and 0.4 μg oligo dT_{12-18} (Pharmacia). The tubes (GeneAmp thin-walled tubes) were incubated at 37°C for 2 min, 400 units of reverse transcriptase (Superscript, BRL) were added, and the tubes were transferred to a 9600 thermocycler. Reverse transcription was conducted for 1 h at 42°C. The samples were then heated for 5 min at 99°C and then placed on ice. At this point the samples were either used directly for PCR or stored at −20°C.

Polymerase Chain Reaction

The reactions were performed in 100 μl of 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 1.45 $MgCl_2$, 0.2 mM each of the four dNTPs, 5 μCi [α-^{32}P]dCTP (sp. act. 3,000 Ci/mmole, Amersham), 2.5 units Amplitaq polymerase (Cetus–Perkin Elmer), 20 pmole (0.4 μM) each of the appropriate 3′ and 5′ primers, and a volume of the reverse transcription reaction that was equivalent to five embryos. In all cases, "Gene-Amp" thin-walled tubes were used and depc-treated water was not used. The primers are listed in Table 1.

The basic PCR program was a 95°C soak for 1 min, followed by a cycle program of 95°C for 10 sec, then 60°C for 15 sec; the last cycle was followed by a 6 min extension at 60°C. For each set of primers two-cell RNA was used to generate semilog plots of the amount of radiolabeled product as a function of cycle number was determined for each set of primers, and all subsequent reactions were conducted in the linear range of such plots. An example for three sets of primers is shown in Figure 1.

Following PCR the tubes were chilled briefly on ice and centrifuged to collect any condensation. Then 25 μl was removed and treated with 2 μl of a 20 μg/ml solution of RNAse A (Worthington) for 10 min at room temperature to digest any remaining carrier RNA. After the addition of 5 μl of 6× loading buffer (0.25% bromophenol blue in 40% sucrose), 13 μl of each sample was subjected to electrophoresis on a 4% agarose gel (Manejwala et al., 1991). The bands were located under UV light and excised with a clean razor blade. The incorporation of radiolabeled deoxycytidine triphosphate (dCTP) in each gel slice was determined by Cerenkov counting. Generation of the diagnostic fragment for each primer set for each cDNA was strictly dependent upon the presence of RNA in the RT reaction, since omitting the RT step did not generate any amplified fragments (data not shown).

Semiquantitative RT-PCR Assay for Specific mRNAs

In our previously reported method to monitor changes in the relative amount of an mRNA (Manejwala et al., 1991), a known amount of α-globin mRNA/embryo was added prior to RNA isolation—this mRNA, which was not present in the embryo at this time, served as an internal standard to correct for differences between samples in RNA recovery and efficiency of the RT-PCR—and both the globin and mRNA under study were coamplified. In the present study, we wanted to analyze the expression of several different mRNAs from a single RNA preparation, and the original coamplification procedure presented three problems. First, the linear range of amplification was unique to each primer pair and was usually different from that of the exogenously added globin mRNA. Second, we observed, as have others (Foley and Engel, 1992), that the relative efficiencies of amplification can differ so greatly between two primer pairs that the presence of one pair inhibits the amplification of the other. Third, the sizes of the amplified globin fragment and that derived from the gene of interest were sometimes too close to allow adequate separation on an agarose gel.

To circumvent these problems, the following method was used. For each experiment involving comparison of changes in mRNA during preimplantation development, the RNA for each stage was isolated concurrently and RT was always performed on 200 embryo equivalents, since we have observed that the efficiency of the RT reaction is highly dependent on the RNA concentration (unpublished observations). Following RT, a mas-

TABLE 1. Primers Used for DNA Sequencing and RT-PCR

Primer	Sequence	Fragment size (bp)[a]
α-globin 5′[b]	5′-GCAGCCACGGTGGCGAGTAT-3′	257
α-globin 3′	5′-GTGGGACAGGAGCTTGAAAT-3′	
β-actin 5′[c]	5′-GTGGGCCGCTCTAGGCACCAA-3′	539
β-actin 3′	5′-CTCTTTGATGTCACGCACGATTTC-3′	
2C#4 5′	5′-TACTCGAGGAGAACCCCTGG-3′	507
2C#4 3′	5′-CCATCCAGAGAGGGAGAGGG-3′	
2C#4 5′[d]	5′-CTGGGAGAATCTCATCCTGG-3′	
2C#4 3′[d]	5′-TAATGTCCTCCTGAAAGCGG-3′	
2C11H 5′	5′-GTTTGATTTTATTGACGATCTGG-3′	313
2C11H 3′	5′-AGGAGCAAAGGGCCACGG-3′	
2A-1 5′	5′-GATCAACTGCGACATCTAGG-3′	216
2A-1 3′	5′-ATTCCAGGTGGTGTCAGAAGG-3′	
2C4D 5′	5′-ATTCTGCCTTGAACTAAATGG-3′	560
2C4D3′	5′-AACAAATAAGCTATAATATGG-3′	
2C4D 5′	5′-CAAAACTAGGATCAGTGTGCC-3′	207
2C4D 3′	5′-AGAAACTGAGTTCTGAACTTCC-3′	
2C7H 5′	5′-ACAGTGGTGTAACTGTGCC-3′	738
2C7H 3′	5′-AAGCCATAGAAATCAGTTATCC-3′	
2C11B 5′	5′-AGACGATGGCCATGAGTGG-3′	1069
2C11B 3′	5′-TAACTAAGACTGCTGACATGG-3′	
2C11B 5′	5′-CTGACTCTACACGATGAGAGG-3′	584
2C11B 3′	5′-TCTTAGGTAAGACATTGAGAGG-3′	
VI12H 5′	5′-AATGCATACACAGAGTGTAGG-3′	280
VI12H 3′	5′-TCCCCTAAAGCAGGTCTGG-3′	

[a]Predicted size in bp of the diagnostic fragment.
[b]The 5′ primer and 3′ primers correspond to bp 241–260 and 555–567, respectively, in the rabbit α-globin genomic clone (Cheng et al., 1986).
[c]The 5′ primer and 3′ primers correspond to bp 25–46 and 540–564, respectively, in the mouse β-actin cDNA clone (Clontech).
[d]Used only for DNA sequencing.

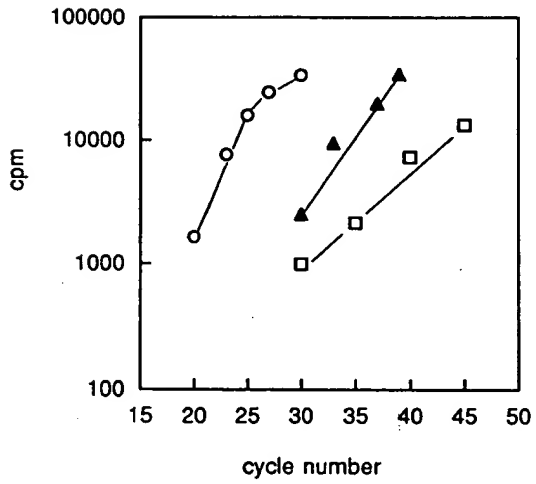

Fig. 1. Relationship between cpm incorporated into the diagnostic fragment and cycle number in RT-PCR. RT was performed as described in Materials and Methods. ○, Globin primers; ▲, 2C#4; □, β-actin.

ter mix for PCR was prepared that contained all the reaction components except the RT and primer. This mix was then subdivided into smaller aliquots, one of which received globin primers, while each of the others received a primer pair specific for one of the genes of interest. These smaller master mixes, now containing gene-specific primers, were aliquoted into thin-walled Gene-Amp tubes. These tubes then received an aliquot of RT reaction corresponding to five embryo equivalents and all the samples were placed in the thermocycler. During the course of the PCR, amplification was stopped after the appropriate number of cycles for each primer set and those samples were removed and placed on ice. This procedure continued until all the samples had been amplified for the requisite number of cycles; then all the tubes were returned to the machine for the final 6 min extension for 60°C. Following agarose gel electrophoresis, the radioactivity in the bands was quantified by Cerenkov counting as described above. For each developmental stage, the ratio of cpm in the band that represented the mRNA of interest versus those in the globin band was calculated. The value for the two-cell embryo was set at 100% and the values of the other stages were expressed relative to it. For each cDNA that was analyzed, the experiment was performed at least three times. Although the temporal pattern of expression was similar for each experiment, the variation in the relative amount of mRNA between experiments prevented pooling of the data. Consequently, results of a representative experiment are shown.

RESULTS

Isolation and Sequencing of cDNA Clones from Two-Cell and Two-Cell Subtraction Libraries

The cDNA clones described in this report were isolated by a method devised to screen for clones containing large open reading frames. Briefly, random clones

TABLE 2. Features of cDNA Clones

Clone	Insert (kb)	ORF (kD)[a]	Poly(A)+[b]	Poly(A)Signal[c]	Class	Library[d]
2C#4	0.984	30	+	+	1	Two-cell
2C11H	0.763	27	+	+	1	Two-cell
2A-1	0.765	15.2	+	+	2	Subtraction
2C4D	0.970	25	+	+	2	Two-cell
2C7H	1.087	28,20,15	+	−	2	Two-cell
2C11B	1.5	16	+	+	2	Two-cell
VI12H	0.541	22	+	+	3	Subtraction

[a]ORF, open reading frame.
[b]+ indicates that a tract of T residues was observed in the 3′ end of the cDNA immediately 5′ to the coding sequence derived from the vector.
[c]+ indicates that the corresponding RNA sequence AAUAAA was observed in the 3′ end of the cDNA.
[d]Subtraction refers to the cDNAs isolated from the two-cell cDNA library that was subtracted against the egg and eight-cell cDNA libraries.

were picked and grown on 96-well microplates, plasmid DNA was prepared on the same plates (Ausubel et al., 1989), and the insert DNA was then amplified by PCR using the pB-FOUT and pB-ROUT primers described under Materials and Methods. Any PCR products >500 base pairs (bp) (the products ranged from about 0.2 kb to 2.7 kb, with an average size of about 1 kb) were then transcribed in vitro using T7 RNA polymerase. A portion of the RNA was then translated in vitro using a rabbit reticulocyte lysate system and the translation products resolved by SDS-PAGE. We screened 96 clones in this manner from each library and analyzed the expression patterns of seven cDNAs.

These seven clones were sequenced and the sequences analyzed using the BLAST algorithm (Altschul et al., 1990). Features of the cDNA clones are found in Table 2 and the results of the data base searches are presented in Table 3. Three of the cDNA clones had no significant match in the data bases, three others had homologies to limited portions of sequences in the data banks, and one encoded *Krev/rap* 1A.

TABLE 3. BLAST Search Results

Clone	Search result
2C#4	90% homology to a 100 nucleotide segment of an *H. sapiens* cDNA clone, FIe035, derived from the human fibroblast line WI-38.[a]
2C11H	No significant sequence homology detected.
2A-1	No significant sequence homology detected.
2C4D	66–76% homology to a 425 nt segment of a *C. elegans* cosmid clone, C30A5 from chromosome III; corresponds to a predicted ORF.[b]
2C7H	90% homology to *Krev/Rap 1A*; 5′ end is missing from clone 2C7H.[c]
2C11B	62–70% homology over the same 70–80 nucleotide region with *X. laevis*[d] and *H. sapiens* nucleolin,[e] and chicken associated microfibril protein.[f]
VI12H	No significant sequence homology detected.

[a]Unpublished sequence, Genbank accession number Z21604 HSAFIE035, submitted by G.J. Arnold and F. Schwager, Genzentrum der Universitat Muenchen, Laboratorium für molekulare Biologie, Am Klopferspitz 18a, 8033 Martinsreid, Germany.
[b]Unpublished sequence, Genbank accession number L10990 CELC30A5, submitted by the Nematode Sequencing Project, Department of Genetics, Washington University, St. Louis, MO 63110 and MRC Laboratory of Molecular Biology, Cambridge CB2 2HQ, England.
[c]Kitayama et al., 1989, Genbank accession number M22995.
[d]Unpublished *X. laevis* nucleolin sequence, EMBL Data Library accession number X72957 XLNUCLEO, submitted by C. Dreyer, Max Planck Institut für Entwicklungsbiologie, Abt Zellbiologie, Spemannstr. 35/V, 7400 Tuebingen, Germany. *X. laevis* cDNA sequence, Rankin et al., 1993, Genbank accession number X63091 XLNUCLEOL.
[e]Srivastava et al., 1990, Genbank accession number M60858 HUMNUCLEOL.
[f]Horrigan et al., 1992, Genbank accession number M76679 CHKASSMIC.

Temporal Pattern of Expression of β-Actin During Preimplantation Development Determined by the RT-PCR Method

Prior to examining the temporal pattern of expression of the different cDNAs by the RT-PCR method, we first confirmed that changes in the pattern of β-actin mRNA detected by this method were similar to those previously determined by Northern and slot blot analyses (Giebelhaus et al., 1985; Paynton et al., 1988; Bachvarova et al., 1989; Taylor and Pikó, 1990). The amount of β-actin decreases during oocyte maturation and continues to decrease up to the end of the two-cell stage. Following ZGA the amount of β-actin increases continuously. As shown in Figure 2, a similar temporal pattern of expression was observed using the RT-PCR method although the absolute magnitude of the increase at the blastula stage was not as great as that measured by hybridization methods; this was likely due to the fact that amount of input actin mRNA was so great at this stage as to take the PCR amplification out of the linear range. This experiment was repeated on three separate occasions with three independently prepared batches of RNA; similar trends were observed in each instance, although the absolute values sometimes differed from one experiment to the next. In each instance, however, the cpm in the globin bands varied by only about 5%, while those in the actin bands changed from stage to stage. The consistency of the number of cpm in the globin strongly suggested that the differences in the actin bands reflected actual differences in

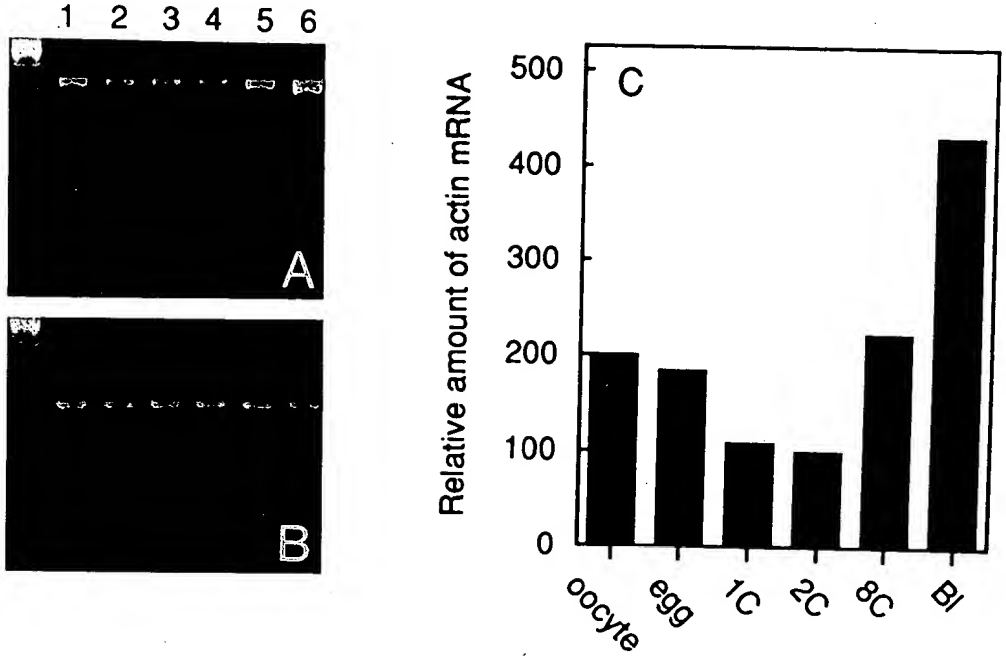

Fig. 2. Changes in the relative levels of β-actin mRNA during oocyte maturation and preimplantation development. **A,** Ethidium-bromide stained agarose gel of the endogenous 539 bp diagnostic β-actin fragment. **B,** Ethidium-bromide stained agarose gel of the exogenous 257 bp α-globin fragment. **Lane 1,** GV-intact oocytes; **lane 2,** ovulated eggs; **lane 3,** one-cell embryos; **lane 4,** two-cell embryos; **lane 5,** eight-cell embryos; **lane 6,** blastocysts. The leftmost unnumbered lane in each gel contains molecular size standards consisting of a 123 bp DNA ladder (Bethesda Research Laboratories); the fastest migrating band corresponds to 123 bp. **C,** Relationship between relative amount of actin mRNA and different developmental stages. In this and in the other figures, the value for the two-cell embryo was set at 100% and the values for the other stages were expressed relative to it.

actin mRNA level rather than variability in recovery or efficiency of RT-PCR.

Temporal Patterns of Expression of Mouse cDNA Clones During Oocyte Maturation and Preimplantation Development

The expression patterns of the clones fell into three distinct groups and an ethidium-bromide stained gel for a representative member of each group is shown in Figure 3. Class 1 clones, of which there were two, displayed expression patterns similar to that of β-actin: a gradual decline during oocyte maturation to the two-cell stage, followed by a rise at the eight-cell and/or blastocyst stages. A representative member of this class, clone 2C#4, is shown in Figure 3A.

The largest class of clones, Class 2, was composed of four clones. Similar to Class 1 genes, the amount of transcript declined during oocyte maturation, but in contrast to Class 1 genes, the RT-PCR signal for Class 2 remained at about the same level in the one-cell embryo as in the unfertilized egg. The signal then decreased from 40–200% between the one- and two-cell stages. These genes also demonstrated an increase in RT-PCR signal in later stage embryos, although for some, the largest relative increase came at the eight-cell stage, whereas in others it did not occur until the blastocyst stage. A representative member of this class, clone 2C4D, is shown in Figure 3B.

Class 3 consisted of a single clone, VI12H (Fig. 3C). This gene showed a large decrease in the RT-PCR signal during oocyte maturation followed by a rise in the signal after fertilization. This peaked at the one-cell stage and then declined sharply by the two-cell stage. In contrast to the clones in Classes 1 and 2, the VI12H gene showed a relatively small increase at the eight-cell and blastocyst stages over the two-cell stage.

The changes in the relative amount of mRNAs for genes in Classes 1 and 3 are shown in histogram form (Fig. 4A and C), and two of the four genes in Class 2 are shown in Figure 4B.

DISCUSSION

We report here the isolation and temporal pattern of expression of seven cDNAs during mouse preimplantation development. Sequence analysis of these seven cDNAs revealed that three cDNAs had no match in the current DNA sequence data banks and three others revealed sequence homology to portions of sequences in the data banks. One cDNA was 90% homologous to the *ras*-related gene *Krev/rap* 1A. In addition, the temporal

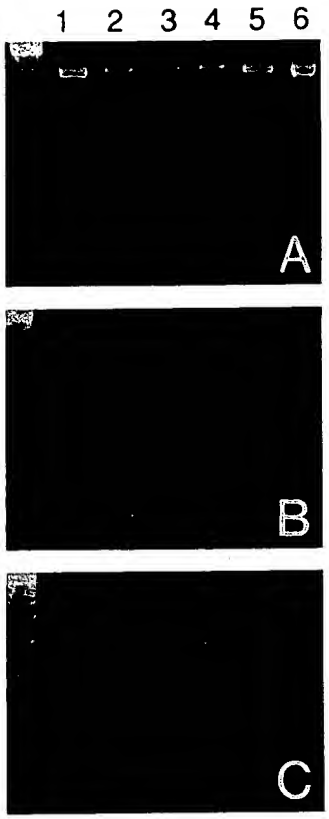

Fig. 3. Ethidium-bromide stained agarose gels showing temporal pattern of expression representative cDNAs. **A,** Class 1, 2C#4; **B,** Class 2, 2C4D; **C,** Class 3, VI12H. **Lane 1,** GV-intact oocytes; **lane 2,** ovulated eggs; **lane 3,** one-cell embryos; **lane 4,** two-cell embryos; **lane 5,** eight-cell embryos; **lane 6,** blastocysts. The leftmost unnumbered lane in each gel contains molecular size standards consisting of a 123 bp DNA ladder (Bethesda Research Laboratories); the fastest migrating band corresponds to 123 bp. The corresponding globin RT-PCR signals for each gene are not shown, but varied by less than 5% of the mean.

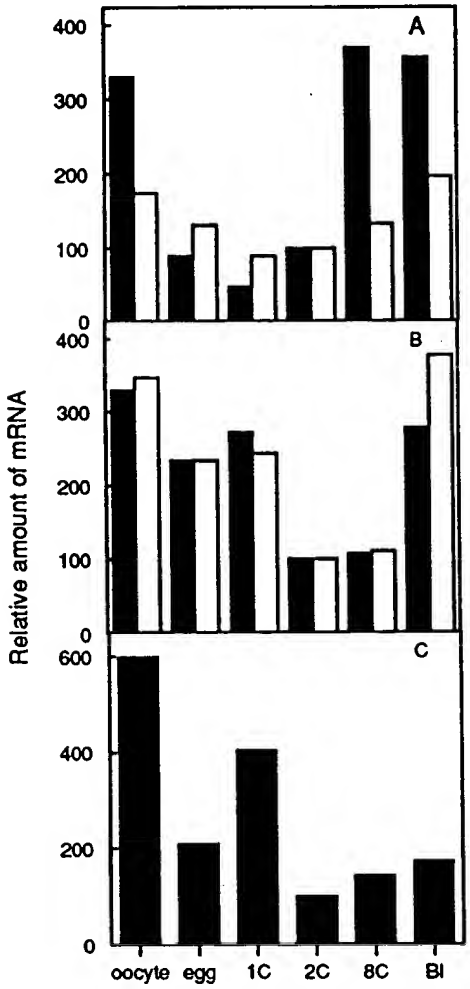

Fig. 4. Changes in the relative levels of representative mRNAs during oocyte maturation and preimplantation development. Ethidium-bromide stained gel bands were excised and radioactivity quantified by Cerenkov counting. The ratios of cpm in the experimental bands versus those in the globin bands were calculated. The value obtained for the two-cell stage was set at 100% and those for the other stages were expressed relative to this to obtain the histograms. **A,** Class 1, 2C#4 (solid bars) and 2C11H (open bars); **B,** Class 2, 2A-1 (solid bars) and 2C4D (open bars); **C,** Class 3, VI12H.

patterns of expression of these cDNAs fell into three categories, two of which have not been reported previously.

We modified an existing RT-PCR assay (Manejwala et al., 1991) that does not entail subcloning in order to increase the number of cDNAs that can be analyzed using a single RNA preparation. Since our objective was to survey as many clones as possible through as many stages as possible, relative quantification was preferable to the more labor-intensive methods of determining absolute mRNA copy number (Becker-André and Hahlbrock, 1989; Gaudette and Crain, 1991). In this regard, the RT-PCR method employed in our studies seems adequate, since it detected changes in the temporal pattern of expression of β-actin that are similar to those made by independent hybridization methods (Giebelhaus et al., 1985; Paynton et al., 1988; Bachvarova et al., 1989; Taylor and Pikó, 1990). This strongly suggests that the temporal patterns of expression observed in Classes 2 and 3, which differ from those in Class 1 (actin-like), are real. It should be emphasized that because the values expressed by the RT-PCR method at each stage are relative and not absolute, no inference can be made about the abundance of any mRNA relative to any other mRNA at a given stage.

The overall expression patterns of the genes in both Classes 1 and 2 are consistent with published reports for total poly(A)$^+$ RNA (Pikó and Clegg, 1982) and for some specific abundant mRNAs (Giebelhaus et al., 1985; Paynton et al., 1988; Taylor and Pikó, 1987; Bachvarova et al., 1989) in which maternal transcripts are degraded by the two-cell stage and are replaced by zygotic transcripts following ZGA such that their amount increases by the eight-cell and/or blastocyst stages. The expression patterns for the two classes dif-

fer significantly, however, during oocyte maturation. In contrast to the Class 1 genes that show a progressive decline during maturation through the one-cell stage, the Class 2 mRNAs persist at high levels (relative to the two-cell stage) up through the one-cell stage and then drop sharply between the one- and two-cell stages. Two-dimensional gel analyses indicate clear differences in patterns of protein synthesis, some of which are due to recruitment of maternal mRNAs, during oocyte maturation and fertilization (Schultz and Wassarman, 1977; Cascio and Wassarman, 1982). It is tempting to suggest that Class 2 mRNAs represent potential maternal transcripts that are recruited for translation and are in fact required during the first cell cycle. Further information about the gene products will be required to address these issues.

The pattern of expression of the gene in Class 3 reveals a transient increase in the one-cell embryo. Two possible mechanisms for this transient increase present themselves. The increase may represent a burst of specific mRNA synthesis and results of recent experiments suggest that the one-cell mouse embryo is transcriptionally competent (Latham et al., 1992; Ram and Schultz, 1993). Alternatively, the increase may reflect changes in the length of the poly(A) tail; all the RT reactions were primed with oligo dT and thus longer tails are likely to result in more efficient cDNA synthesis. Increases in cytoplasmic polyadenylation occur during oocyte maturation and are associated with recruitment on to polysomes and translation (Richter, 1991). The observation that several polypeptides manifest transient changes in their relative rates of synthesis during the one-cell stage is consistent with translational regulation (Latham et al., 1991). An RT-PCR assay for assessing the polyadenylation state of mRNAs (Sallés et al., 1992) could potentially distinguish between de novo mRNA synthesis and readenylation of the pre-existing VI12H mRNA at the one-cell stage.

The GenBank accession numbers for the cDNA clones are: 2A-1, UO1135; V112H, UO1136; 2C#4, UO1138; 2C11B, UO1139; 2C11H, UO1140.

ACKNOWLEDGMENTS

This research was supported by a grant from the NIH (HD 21355 and 22681 to R.M.S.). G.L.T. was supported in part by a training grant from the NIH (T32 HD 07305). J.L.R. was supported by a training grant from the NIH CT32 CA 09140. G.L.T. thanks Lanre Babalola for constructive comments about the manuscript.

REFERENCES

Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropolous MH, Xiao H, Merril CR, Wu A, Olde B, Moreno RF, Kerlavage AR, McCombie WR, Venter JC (1991): Complementary DNA sequencing: Expressed sequence tags and Human Genome Project. Science 252:1651–1656.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990): Basic local alignment search tool. J Mol Biol 215:403–410.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1989): "Current Protocols in Molecular Biology." John Wiley and Sons.

Bachvarova R, DeLeon V (1980): Polyadenylated RNA of mouse ova and loss of maternal RNA in early development. Dev Biol 74:1–8.

Bachvarova R, Cohen EM, De Leon V, Tokunaga K, Sakiyama S, Paynton BV (1989): Amounts and modulation of actin mRNAs in mouse oocytes and embryos. Development 106:561–565.

Becker-André M, Hahlbrock K (1989): Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by *P*CR *a*ided *t*ranscript *t*itration *a*ssay (PATTY). Nucleic Acids Res 17:9437–9446.

Cascio SM, Wassarman PM (1982): Program of early development in the mammal: Post-transcriptional control of a class of proteins synthesized by mouse oocytes and early embryos. Dev Biol 89:397–408.

Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Tores I (1989): An improved culture medium supports development of random-bred-1-cell mouse embryos in vitro. J Reprod Fertil 86:679–688.

Cheng J-F, Raid L, Hardison RC (1986): Isolation and nucleotide sequence of the rabbit globin gene cluster $\psi\zeta$-α1-$\psi\alpha$. Absence of a pair of α-globin genes evolving in concert. J Biol Chem 261:839–848.

Clegg KB, Pikó L (1983a): Poly (A) length, cytoplasmic adenylation and synthesis of poly (A)+ RNA in early mouse embryos. Dev Biol 95:331–341.

Clegg KB, Pikó L (1983b): Quantitative aspects of RNA synthesis and polyadenylation in 1-cell and 2-cell mouse embryos. J Embryol Exp Morphol 74:169–182.

Conover JC, Temeles GL, Zimmermann JW, Burke B, Schultz RM (1991): Stage-specific expression of a family of proteins that are major products of zygotic gene activation in the mouse embryo. Dev Biol 144:392–404.

Endo Y, Schultz RM, Kopf GS (1987): Effects of phorbol esters and a diacylglycerol on mouse eggs: Inhibition of fertilization and modification of the zona pellucida. Dev Biol 119:199–209.

Foley KP, Engel JD (1992): Individual stage selector element mutations lead to reciprocal changes in β- vs. ϵ-globin gene transcription: Genetic confirmation of promoter competition during globin gene switching. Genes Dev 6:730–744.

Gaudette MF, Crain WR (1991): A simple method for quantifying specific mRNAs in small numbers of early mouse embryos. Nucleic Acids Res 19:1879–1884.

Giebelhaus DH, Weitlauf HM, Schultz GA (1985): Actin mRNA content in normal and delayed implanting mouse embryos. Dev Biol 107:407–413.

Horrigan SK, Rich CB, Streeten BW, Li Z-Y, Foster JA (1992): Characterization of an associated microfibril protein through recombinant DNA techniques. J Biol Chem 267:10087–10095.

Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y, Noda M (1989): A *ras*-related gene with transformation suppressor activity. Cell 56: 77–84.

Latham KL, Garrels JI, Chang C, Solter D (1991): Quantitative analysis of protein synthesis in mouse embryos. I. Extensive reprogramming at the one- and two-cell stages. Development 112:921–932.

Latham KL, Solter D, Schultz RM (1992): Acquisition of a transcriptionally permissive state during the 1-cell stage of mouse embryogenesis. Dev Biol 149:457–462.

Manejwala FM, Cragoe EJ, Schultz RM (1989): Blastocoel expansion in the preimplantation mouse embryo: Role of extracellular sodium and chloride and possible apical routes to their entry. Dev Biol 133:210–220.

Manejwala FM, Logan CY, Schultz RM (1991): Regulation of hsp70 mRNA levels during oocyte maturation and zygotic gene activation in the mouse. Dev Biol 144:301–308.

Paynton BV, Rempel R, Bachvarova R (1988): Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. Dev Biol 129:304–314.

Pikó L, Clegg KB (1982): Quantitative changes in total RNA, total poly(A) and ribosomes in early mouse embryos. Dev Biol 89:362–378.

Ram PT, Schultz RM (1993): Reporter gene expression in G2 of the 1-cell mouse embryo. Dev Biol 156:552–556.

Rankin ML, Heine MA, Xiao S, LeBlanc MD, Nelson JW, Dimario PJ (1993): A complete cDNA sequence from *Xenopus laevis*. Nucleic Acids Res 21:169.

Rappolee DA, Brenner CA, Schultz R, Mark D, Werb Z (1988): Developmental expression of PDGF, TGF-α, and TGF-β genes in preimplantation mouse embryos. Science 241:1823–1825.

Richter J (1991): Translational control during early development. BioEssays 13:179–183.

Rothstein JL, Johnson D, DeLoia J, Skowronski J, Solter D, Knowles B (1992): Gene expression during preimplantation mouse development. Genes Dev 6:1190–1201.

Sallés FJ, Darrow AL, O'Connell ML, Strickland S (1992): Isolation of novel murine maternal mRNAs regulated by cytoplasmic polyadenylation. Genes Dev 6:1202–1212.

Schultz RM, Wassarman PM (1977): Specific changes in the pattern of protein synthesis during meiotic maturation of mammalian oocytes in vitro. Proc Natl Acad Sci USA 74:538–541.

Schultz RM, Montgomery RR, Belanoff JR (1983): Regulation of mouse oocyte maturation: Implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. Dev Biol 97:264–274.

Srivastava M, McBride OW, Fleming PJ, Pollard HB, Burns AL (1990): Genomic organization and chromosomal localization of the human nucleolin gene. J Biol Chem 265:14922–14931.

Taylor KD, Pikó L (1987): Patterns of mRNA prevalence and expression of B1 and B2 transcripts in early mouse embryos. Development 101:877–892.

Taylor KD, Pikó L (1990): Quantitative changes in cytoskeletal β- and γ-actin mRNAs and apparent absence of sarcomeric actin gene transcripts in early mouse embryos. Mol Reprod Dev 26:111–121.